Evaluation of Eggshell Quality of Hens Infected with *Salmonella enteritidis* by Application of Compression[1]

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ABSTRACT Eggs collected from hens of different ages and that differed in infection status with *Salmonella enteritidis* were evaluated for the ability to resist cracking following application of maximum compression load from an Instron materials testing machine. Orally infected 24-wk-old hens that were prepeak produced eggs with significantly lower hardness units (HU) of shells compared with a paired control group ($P \leq 0.01$). However, 1 of 3 additional infection trials in hens at peak (29 wk) and older hens postpeak (58 wk) showed an increase in HU in one trial and no difference in the other 2 trials. Thus, *Salmonella enteritidis* may be able to alter HU in a manner that is influenced by multiple factors, which include the age of the hen and the strain used for infection. Hardness was overall a sensitive physiological barometer of age, because readings correlated positively (all $R > 0.50$) with hens entering peak production, regardless of infection status. Detection of a very low HU reading (<1.0) was indicative of a hairline crack in the egg, which increased in incidence from 0.01% preinfection to 0.08% postinfection. Two other clinical signs noted postinfection in hens were that i) daily egg production significantly increased in older hens, and ii) emaciation was evident in a few hens that were infected by contact. These results suggest that there may be supportive approaches to achieve reduction of *S. enteritidis* in table eggs that do not rely on culturing.

Key words: egg quality, laying hen, *Salmonella*, food safety, bacteria

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INTRODUCTION

Salmonella enterica serovar Enteritidis (*S. enteritidis*) is the only one of >2,500 *Salmonella* serotypes that efficiently contaminates the internal contents of the egg following silent infection in the otherwise healthy hen and that results in frequent human illness. Although progress has been made in controlling the problem, the numbers of individual cases have risen from the lowest levels reported for 1998 to 2000; thus, there has not been a significant sustained decline in *S. enteritidis* between the years 1996 to 2003 (CDC, 2003, 2004). England and Wales have also experienced resurgence in outbreaks following several years of vaccination and application of best management practices (O'Brien et al., 2003). It is possible that the decline in incidence that was noted in the late 1990s and attributed to vaccination and better farm management was reversed in 2002. Thus, the epidemiological evidence is that *S. enteritidis* continues to be a major foodborne pathogen that persistently challenges our ability to reduce its incidence. This laboratory conducts research that addresses national needs in regards to development of new methods for detection and control of *S. enteritidis*.

In these experiments, we evaluated compression load as a method for detecting subtle change that might occur in eggshell quality following infection of hens with *Salmonella enteritidis*. This approach originates from previous research, which showed that some strains of *S. enteritidis* that produce high-molecular-mass lipopolysaccharide (**HMM LPS**) could alter the reproductive tract function of hens under certain circumstances (Parker et al., 2001). Lipopolysaccharide is a well-characterized toxin that exerts a powerful effect on host cells (Holst et al., 1996; Heine et al., 2001). Although chickens are unusually resistant to the deleterious effects of LPS compared with some mammals, HMM LPS induced involution and regression of the hen's reproductive tract at subcutaneous infectious dosages of 10^8 cfu (Parker et al., 2002). During these experiments, eggs were observed that were softer than usual and that sometimes lacked a calcified shell. However, these artificially high dose studies were not indicative of what might happen in a production setting where infection is more likely to be contracted at low dosages and by a mucosal route. Thus, the objectives of this research were to assess if a compression load could be used to detect changes in the shell quality of eggs obtained from

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hens that varied in age and by infection status. A supporting objective was to observe hens for any other subtle sign of infection that might be used by producers as an indicator of flock infection status.

MATERIALS AND METHODS

Strains and Media

The strains used in these experiments originate from *S. enteritidis* strain SE6, which has been shown in a number of investigations to produce high-incidence egg contamination (Gast and Beard, 1990; Petter, 1993; Gast et al., 2002;). Strain ESQRU 21060, which was isolated in 2001 from the albumen of a contaminated egg, was used to infect seeder hens by oral gavage. It is a first-passage wild type isolate from experiments that used ESQRU 21000 to initiate infection (Gast et al., 2002).

In Experiment 2, hens were infected with 1 of 3 characterized strains of *S. enteritidis*. These strains were a) ESQRU 21000, which is a designated wild type strain that produces HMM LPS (Parker et al., 2001); b) ESQRU 20154, which is a wzz (O-chain length determinant of LPS) mutant (**wzz**$^{-}$) that produces low-molecular-mass LPS but not HMM LPS (Parker et al., 2001); and c) ESQRU 21001, which is an flhD (master global regulator subunit of flagellation) mutant (**flhD**$^{-}$) that cannot produce flagella and is not motile (Parker and Guard-Petter, 2001). All 3 strains grow to high cell density and have been used previously to produce high-incidence egg contamination (Guard-Petter, 1998).

To obtain targeted dosages, serial 10-fold dilutions in 1× PBS were made of each strain that had been grown for 16 h with no shaking at 37°C in brain-heart infusion broth. Final dose/hen (cfu/hen) was determined from dilutions plated on brilliant green agar (Becton Dickinson, Cockeysville, MD). Experimental rooms were assayed once immediately before infection for being free of *Salmonella* by culturing 12 samples of droppings collected from hens that had been in the rooms for at least 2 wk. Briefly, approximately 1 g of chicken droppings was collected from 6 different sites in experimental rooms and incubated in 10 mL of brain-heart infusion broth for 48 h at 37°C. One milliliter of culture was transferred to Rappaport Vassiliadis enrichment broth and incubated for 24 h (Parker et al., 2001). Culture was used to inoculate brilliant green agar for isolation of colonies and it was incubated for 24 h at 37°C. Suspect colonies that appeared to be *Salmonella* were further characterized by performing a series of biochemical reactions (Enterotube, Becton Dickinson) to confirm bacterial genus. Final identification of serotype was made by detection of O-factors 9, 12 (Difco, Detroit, MI) by slide agglutination and by detection of motility (Guard-Petter, 1997). All 12 environmental samples were negative for *Salmonella* of any serotype before infection of hens.

Care and Use of Hens in Research

All animal experiments were conducted following review and approval by the Animal Care and Use Committee located at the Southeast Poultry Research Laboratory, which uses protocols developed in accordance with Public Health Service/Office of Laboratory Animal Welfare guidelines filed under assurance number A4298-01. Single Comb White Leghorns were procured from specific-pathogen-free flocks raised on-site in segregated forced-air positive-pressure housing. Hens that were moved to experimental rooms were housed in individual layer cages, and fed and watered ad libitum. Standard operating procedure included a photoperiod of 14L:10D.

Eggs in Experiment 1 were collected daily for 55 d from 24-wk-old hens that had been divided into 2 experimental groups of 24 and 25 birds each. Infection was initiated on d 32 of housing oral gavage of 6 hens with 6.5×10^5 cfu of ESQRU *S. enteritidis* strain 21060 in one of the 2 rooms. These hens were placed back into cages and the infection was allowed to spread by contact to the remaining 18 hens. These seeder hens were spaced so that 2 or 3 uninfected hens were left to be contact-infected on either side. The other 25 flock mates were housed separately as uninfected controls. Eggs for analysis were tested for shell quality for 3 wk before and after infection. Eggs were cultured for 3 wk after infection. Data were separated into sets collected before and after the start of infection and according to the infection status of the hens (uninfected, infected by oral gavage, or infected by contact).

Hens in Experiment 2 were divided into 3 groups that included 23 to 26 mature hens (29 wk old) and between 5 and 6 aged hens (58 wk old) per group. Infection of seeder hens in Experiment 2 was initiated in each room by injecting 6 hens i.v. with 10^6 cfu of the respective strain. Infected hens were distributed between 2 rows so that each infected hen was flanked by 1 or 2 uninfected hens. Eggs were collected for analysis of shell quality and determination of egg production from all hens for 2 wk before and 3 wk after infection. Eggs were cultured only from the 29-wk-old infected hens, because too few older hens were available to establish the presence of high-incidence egg contamination.

A minimum of 100 eggs were required for culturing from any one experimental group, because this is the minimum number required that allows detection of high-incidence egg contamination. High-incidence egg contamination is defined as recovery of 1% of contaminated eggs during the 21 d postinfection. It was not possible to rule out the presence of low-incidence egg contamination in experimental settings, because that would require culturing more than 20,000 eggs from each group. Eggs were evaluated for the presence of preexisting cracks by visual examination but not by candling, because these facilities were not available in experimental rooms used to house infected hens. Obviously cracked eggs were not included in testing and were not cultured, because they may have become contaminated due to eggshell penetration.

Measurement of Shell Quality by Compression Load

Eggshell quality was assessed using an Instron Universal Testing machine model 5500R (Instron Engineering

Table 1. Average hardness units of eggshells obtained before and after infection of 24-wk-old hens with *Salmonella enteritidis*

Experimental group	No. of hens	No. of eggs	Average daily hardness (95% CI)	*P*-value (groups compared)
1. Uninfected control hens (wk 1 to 3)	25	431	3.370 (0.062)	0.31 (1 vs. 2)[a]
2. Uninfected control hens (wk 4 to 6)	25	420	3.347 (0.063)	—
3. Hens before infection (wk 1 to 3)[1]	24	411	3.401 (0.068)	0.20 (3 vs. 4)[a]
4. Hens after infection (wk 4 to 6)	24	400	3.359 (0.070)	—
5. Hens for c.i., pre-infection (wk 1 to 3)	18	307	3.363 (0.078)	0.29 (5 vs. 6)[a]
6. Hens for c.i., post-infection (wk 4 to 6)	18	297	3.393 (0.073)	—
7. Hens for o.g., pre-infection (wk 1 to 3)	6	104	3.514 (0.140)	0.01 (7 vs. 8)[b]
8. Hens for o.g., post-infection (wk 4 to 6)	6	103	3.258 (0.174)	—

[a,b]Means of groups compared are either (a) similar or (b) significantly different ($P \leq 0.05$).

[1]Groups 3 and 4 include both groups of hens that were infected by contact (c.i.; groups 5 and 6) or by oral gavage (o.g.; groups 7 and 8).

Corp., Canton, MA; Strong, 1989). Data was measured as kilograms force (kgf) needed to introduce a crack into the shell, also termed hardness units (**HU**). The load cell delivered 509.86 kgf maximum and was internally calibrated before each day of data collection. Crosshead speed was set at 60 mm/min, with a programmed automatic stop set when a 40% reduction of the maximum load had occurred. These parameters produced a hairline crack in the egg but no loss of egg contents, because the inner shell membrane remained intact.

Culture of Salmonella *from Eggs*

Whole eggs were cultured as described previously (Gast and Beard, 1990). Briefly, eggs were dipped in 1.5% Lugol's solution of iodine 5 times, air dried, and then individually cracked over the edge of a beaker covered with a sterile strip of aluminum foil. Whole egg contents were dropped into sterile stomacher bags, mixed for 3 min, and then transferred into specimen cups containing 160 mL of tryptic soy broth and 35 ppm of $FeSO_4$. Contents were incubated at 37°C for 48 h. One milliliter of culture was then transferred to 10 mL of Rappaport Vassiliadis enrichment broth, and incubated for 24 h. Culture was streaked for isolation of colonies on brilliant green agar and incubated. Colonies of *Salmonella* were further identified and serotyped as previously described.

Statistical Analyses

The Student's *t*-test was used to determine the probability (*P*-value) that shell quality and daily egg production were not altered by age or infection status; *P*-values of 0.01 or less were defined as significant and *P*-values of between 0.05 and 0.01 were defined as possibly significant. Data points were entered into a scatter graph and then analyzed by least squares equations to determine the best curve fit. The fourth, fifth, and sixth polynomial equations were examined, as well as linear, log, exponential, and spline functions. A positive determinant of fit (Rval) was defined as >0.50, with 1.0 defining a perfect fit for all data. Statistical analysis software available through Slidewrite Version 6 (Advanced Graphics Software, Inc, Encinitas, CA) was used to conduct curvilinear analysis.

RESULTS

Experiment 1. Initiation of Flock Infection by Oral Inoculation of Young Hens

All eggs collected from 24-wk-old orally infected hens and from the hens in the same room that had been left to be infected by contact had negative cultures. However, the average HU of shells collected from the 6 orally infected hens was significantly different before and after infection (Table 1). The average HU of eggs from orally infected hens was insignificantly lower than the average for control hens ($P = 0.13$). Hardness unit averages for eggs collected before and after infection from contact-infected hens were no different ($P = 0.29$), nor were they different from HU averages for the uninfected control hens that were housed separately ($P = 0.31$; Table 1). Orally infected hens had a higher standard deviation after infection compared with other groups, but this group included fewer hens. These results suggest that infection of young hens with *S. enteritidis* decreased eggshell quality in the absence of detectable egg contamination.

Necropsy Results. Two of the 6 orally infected hens had observable gross pathology. Both hens were emaciated as evidenced by atrophy of breast muscle and by the concomitant presence of a prominent keel bone. One of these hens had inflammatory peritonitis that was not due to rupture of an ovarian follicle, which is a commonly encountered reproductive problem in the laying hen. The second orally infected hen had cystic development of the residual Müllerian duct, which is the embryonic right oviduct that normally does not develop in the hen (this pathology had been noted to occur in previous studies that used histology to confirm the nature of the cystic structure). In addition to the hens that were orally infected, 4 of the 18 hens that had been left to become contact-infected had gross pathology. Two were emaciated (one with peritonitis), one had peritonitis without emaciation, and one had a regressed (nonfunctioning) ovary. None of the 25 control hens that had been housed in a different experimental room had any of these lesions. In total, 4 of 24 hens within the room containing infected hens (16.7%) were emaciated.

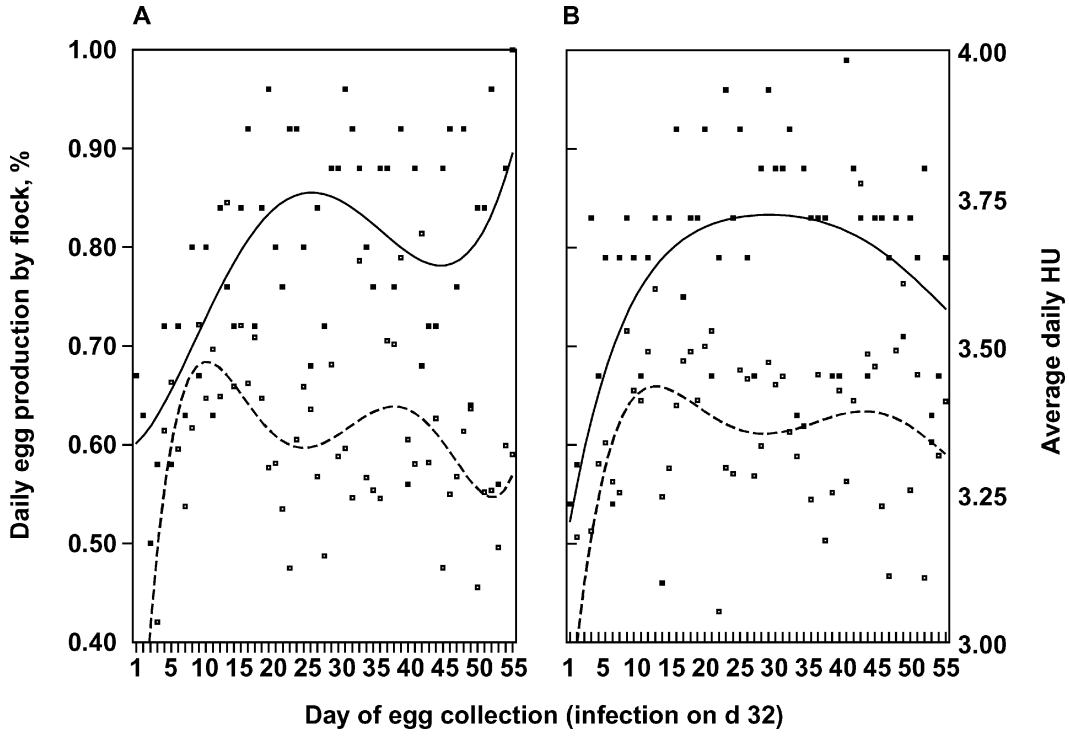

Figure 1. Correlation of shell hardness with daily egg production of young hens. Results from uninfected control hens and hens infected at d 32 of data collection are shown in graphs A and B, respectively. Eggs were collected for 55 d and analyzed by compression load to generate hardness units (HU) of eggs using an Instron materials testing machine. Solid squares (■) are data points for daily egg production by a treatment group, and open squares (□) are the daily average HU for that group of hens. The solid line (——) shows the curvilinear plot (fifth polynomial) of daily egg production; the dashed line (- - -) is the curvilinear plot (fifth polynomial) of average daily HU.

Other Findings in Young Hens. Data were collected for 55 d on daily egg production and on the average daily HU of eggs. These data were plotted as a scatter graph and were further analyzed by curvilinear analysis, which helps define patterns of data distribution that suggest biological trends. Results from application of the fifth polynomial equation to egg production data showed that 24-wk-old hens in the control group (Figure 1A) and in hens infected at d 32 of data collection (Figure 1B) were still young and had not yet reached peak production. In this laboratory, peak production is defined as at least 80% of hens producing an average of 1 egg/d for 3 consecutive days. The uninfected and infected flocks reached peak production on d 16 and 9, respectively (Figure 1, A and B).

Hardness units increased in young hens until peak production was reached, but then leveled off and thereafter underwent periodic fluctuations in both uninfected and infected hens (Figure 1, A and B). Infected hens (Figure 1B) had a different pattern than uninfected hens, which suggests that the relationship between production and HU may have changed following infection at d 32 as compared with controls (Figure 1, A and B).

Experiment 2. Initiation of Flock Infection by i.v. Injection of Mature Hens

In the second experiment, hens of mixed ages were either exposed to wild type *S. enteritidis*, wzz *S. enteritidis* (wzz$^-$), or flhD *S. enteritidis* (flhD$^-$). The treatment groups consisted of 26, 26, and 23 young hens (29 wk old) and 6 aged hens (58 wk old) in each room, respectively. Infection was initiated intravenously by injecting 6 of the younger hens in each group with 10^6 cfu, which left the remainder of the hens to contract infection by contact.

Culture Results. Egg contamination occurred at high incidence for the 3 groups of infected younger hens and it was, overall, 1.72% (22 of 1277 total eggs collected). Too few eggs were collected from older hens to warrant culture, as discussed in Materials and Methods. Egg contamination incidences within the 29-wk-old hens (contact-infected plus injected hens) were 1) 3.25% (14/431 eggs) for hens infected with wild type *S. enteritidis*, 2) 0.87% (4/460) for hens infected with wzz$^-$ *S. enteritidis*, and 3) 1.04% (4/386) for hens infected with flhD$^-$ *S. enteritidis* (Table 2). Table 2 also shows production results for hens that were contact infected. In contact-infected hens, wzz$^-$ appeared to have a reduced ability to cause egg contamination, because egg contamination dropped from 0.87 to 0.27% once eggs collected from i.v.-infected hens were excluded from calculations (Table 2). In contrast, there was little difference in the incidence of egg contamination for hens that had been i.v.- or contact-infected with wild type and flhD$^-$ strains (Table 2). These results suggest that HMM LPS, but not flagellation, contributes to the incidence of egg contamination, which is in agreement with previous assessments of the virulence properties of these strains.

Statistical Analysis of Shell Quality from 29- and 58-Wk-Old Hens. The averaged HU before and after infec-

Table 2. Flock production characteristics and contamination in eggs of hens infected with *Salmonella enteritidis*

Strain	No. of hens	% flock daily egg production[1] pre-/postinfection (95% CI)	% eggs contaminated (all hens)[2]	% eggs contaminated (c.i. hens only)
Mature hens (29 wk old)				
Wild type	26	83.5 (1.47) / 78.9 (1.75)[a]	3.25 (14/431)	3.13 (11/351)
wzz^{-3}	26	80.8 (2.42) / 84.4 (1.72)[a]	0.87 (4/460)	0.27 (1/360)
$flhD^{-3}$	23	84.5 (2.50) / 79.9 (1.94)[a]	1.04 (4/386)	0.99 (3/303)
Aged hens (58 wk old)				
Wild type	6	70.2 (0.72) / 76.7 (0.56)[b]	NC[4]	NC
wzz^-	5	64.3 (0.47) / 69.5 (0.46)[b]	NC	NC
$flhD^-$	6	66.7 (0.80) / 74.6 (0.47)[b]	NC	NC

[a,b]Same (a) or different (b) means pre-/postinfection, respectively ($P < 0.05$).

[1]CI = confidence interval.

[2]Includes data from 6 hens infected i.v. that exposed other hens to contact infection (c.i.).

[3]wzz^- = a wzz [O-chain length determinant of lipopolysaccharide (LPS)] mutant that produces low-molecular-mass LPS but not high-molecular-mass LPS; $flhD^-$ = f1hD (master global regulator subunit of flagellation) mutant that cannot produce flagella and is not motile.

[4]NC = not cultured; too few eggs were obtained for determination of high-incidence contamination.

tion for each of 3 treatment groups are shown in Table 3. The most significant finding was that 59-wk-old hens that were past peak production had shells with significantly lower HU than those observed in 29-wk-old hens that were at peak of production. This finding was expected, as will be discussed, and it confirms that this methodology is in agreement with findings from other investigations. Infection did not alter HU significantly except for hens that had been infected with the wzz^- strain, which had previously been observed to cause more gross pathology in the reproductive tract of producing hens than did the wild type strain (Parker et al., 2001). Whereas mature hens significantly increased HU ($P \leq 0.01$) following infection with the wzz^- strain, aged hens tended to have a decrease in HU that was not quite statistically significant ($P \leq 0.07$; Table 3). When considered together with results obtained from 24-wk-old hens that were prepeak production, it appears that *S. enteritidis* infection can, at times, significantly alter HU. These results support the concept that *S. enteritidis* interacts with the hen's reproductive tract at low dosages as well as high, but that variation in results between experiments will be encountered.

DISCUSSION

This research project originated from discussions with representatives from the Food and Drug Administration, who encouraged the evaluation of flock infection status with methods that did not rely on handling or culture of eggs. It is unlikely that culturing of eggs will ever be discontinued in the efforts to decrease *S. enteritidis* in the egg supply. However, it might be possible to refine machinery to better target batches of eggs that should be cultured before their entry into market. For example, if the incidence of hairline cracks does increase above baseline, then in-line equipment that detects hairline cracks could be used to remove specific batches of eggs for culturing or diversion to pasteurization. Such a high throughput approach might have the benefit of removing product at risk from ever entering the market. An increase in incidence of hairline cracks even without an otherwise general decline in shell quality following infection of hens with *S. enteritidis* could be a substantial hazard to the public health (Todd, 1996).

A physical sciences approach to improving egg safety offers significant advantages over microbiological or mo-

Table 3. Hardness characteristics of eggs collected from hens pre- and postinfection with *Salmonella enteritidis*[1]

Strain	No. of eggs assayed pre-/postinfection	Mean hardness (95% CI) pre-/postinfection[2]	No. of cracked eggs pre-/postinfection	*P*-value of means pre-/postinfection
29-wk-old hens at peak production				
Wild type	279/417	3.63 (0.404) / 3.59 (0.306)[a]	0/1	0.27
wzz^{-3}	292/439	3.54 (0.400) / 3.67 (0.324)[b]	0/4	≤0.01
$flhD^{-3}$	273/372	3.78 (0.417) / 3.80 (0.325)[a]	0/4	0.14
58-wk-old hens post peak production				
Wild type	65/103	3.14 (0.350) / 3.23 (0.297)[a]	0/1	0.20
wzz^-	74/68	3.29 (0.491) / 3.01 (0.399)[a]	1/2	0.07
$flhD^-$	58/95	3.42 (0.408) / 3.46 (0.318)[a]	0/0	0.36

[a,b]Means of hardness are either (a) similar or (b) significantly different ($P \leq 0.05$).

[1]The mean hardness units of eggs from 29- vs. 58-wk-old hens was significantly different both pre- and postinfection for all 3 strains.

[2]Means are measured in hardness units (HU).

[3]wzz^- = a wzz [O-chain length determinant of lipopolysaccharide (LPS)] mutant that produces low-molecular mass LPS but not high-molecular-mass LPS; $flhD^-$ = f1hD (master global regulator subunit of flagellation) mutant that cannot produce flagella and is not motile.

lecular detection methods, because the latter are labor intensive, costly to use as screening tools in a food safety setting, and not as amenable to automation. Results from these experiments suggest that compressing eggs easily detects hairline cracks, which appeared to increase in incidence following infection. Spectrophotometry, light transmittance (including candling), and ultrasound are other methods that could be developed further to detect hairline cracks in eggs that escape detection by visual inspection (De Ketelaere et al., 2002; Liu et al., 2003; Stojanoff et al., 2004). It should be considered that "check detection" equipment that is already used to screen eggs for cracks could be recalibrated to increase the stringency at which shells are monitored.

Compression load was a sensitive method for evaluating the age and production status of the hen. Thus, this research supports previous findings that compression load is a useful research tool for investigation of eggshell quality (Strong, 1989), and that shell quality is influenced by age (al-Batshan et al., 1994; Bar et al., 1998; De Ketelaere et al., 2002). Compression load might not be measuring the physical component of the eggshell that is most directly altered by infection or by age. It is possible that "brittleness" increases postinfection or with age, although the physics of brittleness are not defined (Fernandez et al., 1997). It is possible for compression load to pick out eggs without destroying them if load parameters are adjusted to deliver a minimal maximum load rather than a maximal load to the point of failure. Such an adjustment might allow the majority of eggs to pass inspection without concern for rupture of the shell.

The inability of producers to survey flocks daily for signs of recently acquired infection by *S. enteritidis* impedes control of the disease in humans. Results here suggest that there are some subtle signs of infection in hens that occur following low-dose contact exposure. One of the more easily detected signs was emaciation, which occurred in hens that were contact-exposed and in hens that had been infected by oral gavage at the artificially high challenge dose in the range of 10^5 to 10^6 cfu. This result suggests that birds in densely populated hen houses might also develop emaciation, even if the initial infecting dose is low or variable between hens.

Further analysis of how infection of hens by *S. enteritidis* correlates with subtle signs in exposed flocks requires evaluation in production settings, because eggshell quality, bird health, and management practices are integrated parts of a complex system. For example, many infectious diseases in hens can change shell quality, but usually these result in loss of production (i.e., egg drop syndrome, Newcastle disease virus, and avian influenza; McFerran, 1979; Alexander, 2000; Henzler et al., 2003). In addition, egg production is influenced by many noninfectious factors, such as nutrition. Evaluation of best management practices intended to improve public health requires recognition of the complexity of a production system that brings to market billions of eggs per year (Anonymous, 1998; Guard-Petter, 2001).

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